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Dual Role for N^2-Acetylornithine 5-Aminotransferase from *Pseudomonas aeruginosa* in Arginine Biosynthesis and Arginine Catabolism

RICHARD VOELLMY AND THOMAS LEISINGER*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Zürich, Switzerland

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In *Pseudomonas aeruginosa* N^2-acetylornithine 5-aminotransferase (ACOAT), the fourth enzyme of arginine biosynthesis is induced about 15-fold by cultivating the organism on a medium with L-arginine as the sole carbon and nitrogen source. Synthesis of the enzyme is subject to catabolite repression by a variey of carbon sources. ACOAT from strain PAO 1 was purified over 40-fold to electrophoretic homogeneity. A molecular weight of approximately 110,000 was obtained by thin-layer gel filtration. Electrophoresis in sodium dodecyl sulfate gels gave a single band corresponding to a molecular weight of 55,000. Purified ACOAT catalyzes the transamination of N^2-acetyl-L-ornithine as well as of L-ornithine with 2-oxoglutarate (K_m values of 1.1, 10.0, and 0.7 mM, respectively). With N^2-acetyl-L-ornithine as amino donor, the pH-optimum of the enzymatic reaction is 8.5; with L-ornithine as amino donor, 9.5. The catalytic properties of ACOAT as well as the regulation of its synthesis indicate that in *P. aeruginosa* this enzyme functions in the biosynthesis as well as in the catabolism of L-arginine.

In *Pseudomonas aeruginosa*, as well as in all other microorganisms studied, arginine biosynthesis proceeds from glutamate via ornithine to arginine. The first four intermediates of the eight-step pathway are N^2-acetylated (14, 30). In *P. aeruginosa* the levels of three out of eight arginine biosynthetic enzymes tested responded to changes in the extracellular arginine supply. Ornithine carbamoyltransferase (EC 2.1.3.3; 14) and N^2-acetylglutamate 5-semialdehyde dehydrogenase (EC 1.2.1.38) were repressed, whereas N^2-acetylornithine 5-aminotransferase (ACOAT, EC 2.6.1.11) was induced by arginine (R. Voellmy and T. Leisinger. J. Gen. Microbiol. **73**:xiii, 1972).

A similar induction of ACOAT by arginine has been observed in *Pseudomonas putida* (I. N. Chou and I. C. Gunsalus. Bacteriol. Proc., p. 162, 1971). Induction of a biosynthetic enzyme by the end product of a pathway represents an unusual regulatory pattern and prompted us to investigate the role of ACOAT in the arginine metabolism of *P. aeruginosa*. The explanation for the induction of ACOAT offered in this report is based on the substrate specificity of the purified enzyme. It will be shown that ACOAT catalyzes the transamination of N^2-acetyl-L-ornithine as well as of L-ornithine. As the former reaction is a step in arginine biosynthesis and the latter a reaction of arginine catabolism (3, 8, 22, 29), the regulation of the synthesis of ACOAT apparently is in accordance with its function in arginine catabolism. A preliminary report of some of these results has appeared elsewhere (31).

MATERIALS AND METHODS

Organisms and cultural methods. *P. aeruginosa* PAO 1, a wild-type strain (ATCC 15692), as well as PAO831 (*ade-66, his-151, ura-21, thi-1, pro-71, ese-14*, FP$^-$), PAO 853 (*ade-66, his-151, ura-21, thi-1, pro-70, ese-14*, FP$^-$), and PAO 879 (*ade-136, leu-8, pro-73, ese-20, chl-2*, FP$^-$) were obtained from B. Holloway. For regulation studies the bacteria were grown in Fernbach flasks at 37 C on minimal medium P containing either L-glutamate, putrescine (1,4-diaminobutane), or L-arginine, each at 20 mM, as the only carbon and nitrogen source (12). Bacterial growth was followed by measuring the absorbance at 546 nm in an Eppendorf photometer. Large scale cultivation of strain PAO 1 was done in a F 0300 fermentor (Chemap AG, Männedorf, Switzerland) on 250 liters of medium P containing all components at double concentration (D. Haas and T. Leisinger, Eur. J. Biochem., in press). Growth of the organism in the fermentor was optimal when aeration was discontinuous, i.e., when the medium was saturated with oxygen by pulses of aeration in 30-min intervals. The cell paste obtained was stored at −20 C for longer than 12 months without decrease in the specific activity of ACOAT.

Preparation of cell extracts. The cell paste obtained from a 1-liter Fernbach culture was suspended in 0.1 M potassium phosphate, pH 7.0, at 15% (wt/vol) and subjected to sonic disruption in an MSE 150-W Sonifier by means of eight bursts of 15 s each. Crude extracts were obtained after centrifugation of the suspension at 20,000 × *g* for 60 min at 4 C. Dialysis of the crude extracts as well as the addition of pyridoxal-5'-phosphate even to dilute extracts did not affect ACOAT activity. ACOAT was stable in these extracts for a minimum of several days.

Enzyme assays. ACOAT was assayed according to Albrecht and Vogel (1) with the following modifications. The incubation mixture consisted of 100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 9, at 25 C, 15 mM N^2-acetyl-L-ornithine, 15 mM 2-oxoglutarate, 0.075 mM pyridoxal-5'-phosphate, and 0.1 ml of enzyme in a final volume of 0.5 ml. The pH of the reaction mixture containing all components except the enzyme was adjusted to 8.5 at 25 C. The reaction was started by the addition of prewarmed reaction mixture to the enzyme. After 5 to 30 min of incubation at 37 C, the reaction was stopped by the addition of 0.2 ml of 10.3 M HCl. The incubation tubes were then stoppered and kept for 45 min in a boiling-water bath. Subsequently the incubation mixture was cooled to room temperature and 1.4 ml of 3.6 M sodium acetate and 0.2 ml of a 33 mM solution of ortho-aminobenzaldehyde were added for color development. Absorbance was read in a Eppendorf photometer at 436 nm. For the assay of ornithine 5-aminotransferase (OAT) this procedure was modified in the following way: 15 mM N^2-acetyl-L-ornithine was replaced by 50 mM L-ornithine. The pH of the reaction mixture containing all components except enzyme was adjusted to 9.0 at 25 C. The boiling step was omitted. Putrescine aminotransferase was measured as ACOAT, but with 25 mM putrescine as amino donor and 100 mM glycine-NaOH (pH 10.5 at 25 C) as buffer. The pH of the reaction mixture was set to 10.5 at 25 C. The boiling step was omitted. Enzyme activities were a linear function of both incubation time (up to 30 min) and protein concentration up to an absorbance of 0.3 in the Eppendorf photometer at 436 nm. For ACOAT and OAT, 1 U is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product per min under assay conditions.

Calculation of enzyme units is based on an absorption coefficient of the colored derivative of L-glutamate 5-semialdehyde of $1.9 \times 10^3\ M^{-1}\ cm^{-1}$ at 436 nm and room temperature, as determined by converting known amounts of [1-^{14}C]2-oxoglutarate and N^2-acetyl-L-[U-^{14}C]ornithine to L-[1-^{14}C]glutamate and to N^2-acetyl-L-[U-^{14}C]glutamate 5-semialdehyde with purified enzyme. The extinction coefficient in the incubation mixture at 436 nm of the colored derivative of the product formed in the putrescine aminotransferase reaction is unknown. Therefore 1 U of putrescine aminotransferase is defined as the amount of enzyme which leads to the formation of an absorbance at 436 nm of 0.1 per h under the assay conditions described.

Protein determination. Protein was estimated by the method of Lowry et al. (18) with bovine serum albumin as a standard.

High-voltage paper electrophoresis. ^{14}C-labeled substrates and products of aminotransferase reactions were separated by high-voltage paper electrophoresis in 0.75 M formic acid at pH 2.0 (11). A detailed description of the application of this method to the separation and quantitative determination of the different substrates and reaction products relevant in this paper will be presented in a future communication (R. Voellmy and T. Leisinger, manuscript in preparation).

Polyacrylamide gel electrophoresis. Disc gel electrophoresis was performed according to Davis (7). Analytical electrophoresis was done with 7.5% acrylamide gels (5.5 by 0.5 cm) at 4 C and a current of 3 mA per gel. Samples (10 to 50 μl) containing approximately 10 μg of protein were applied. The gels were stained with Coomassie brilliant blue R-250, destained as described (36), and scanned at 600 nm. For the analysis of aminotransferase activity in gels (0.01 U of OAT per gel), they were incubated after electrophoresis for 15 min at room temperature in 0.1 M potassium phosphate (pH 7.0) containing 1 mM pyridoxal-5'-phosphate. ACOAT activity was localized in the gels by preparing 3-mm slices which were assayed for enzymatic activity in the standard ACOAT assay. After incubation at 37 C the gel slices were removed from the reaction mixtures. OAT activity was localized in situ by incubation of the gels for 15 to 30 min at 37 C in a mixture of 8 ml of OAT reaction mixture and 2 ml of 33 mM ortho-aminobenzaldehyde. Sodium dodecyl sulfate gel electrophoresis was carried out as described (36),using 0.08 M potassium phosphate as gel buffer (electrophoresis time, 90 min). Approximately 10 μg of protein was applied in a volume of 10 to 50 μl to 7.5% acrylamide gels.

Gel electrofocusing. Electrofocusing in 7.5% acrylamide gels (5.5 by 0.5 cm) was done as described (37), covering a pH range between 3.0 and 10.0 Protein precipitaion was recorded by scanning the gels at 500 nm.

Thin-layer gel filtration. The method used is a modification of the procedures described (24, 25). Two glass plates (20 by 20 cm) coated with a 0.8-mm layer of Sephadex G-200 Superfine were placed in a Pharmacia thin-layer gel apparatus and connected with the buffer reservoirs by filter paper wicks (Whatman 3MM). The layers were equilibrated with running buffer (0.1 M potassium phosphate, pH 7.0, with or without 0.5 M NaCl or 0.1 M Tris-hydrochloride, pH 9.0, at 25 C) overnight at room temperature and at an inclination of 15°. Samples of rabbit muscle aldolase, bovine serum albumin, hen ovalbumin, and ferritin as front dye (20 to 40 μg each) were applied in 2 to 10 μl of running buffer. Cytochrome *c* (20 μg) from horse heart and human hemoglobin (20 μg) A were applied in 5 μl of running buffer.

An experiment consisted of the analysis of two plates run in parallel at room temperature and at an inclination angle of 15° for about 3 h. One plate contained all marker proteins; the other contained only the colored markers and several sample spots with 0.01 U of OAT in 10 μl. Immediately after the

experiment the migration distances of the colored markers were measured on both plates. The colorless markers on the marker plate were stained by incubation for 15 to 30 min in an iodine atmosphere. OAT activity on the sample plate was detected by activity staining in the following way. The wet plate was prewarmed to approximately 37 C, by mounting it above a water bath, and was sprayed with a solution of 5 mM pyridoxal-5'-phosphate in 0.1 M Tris-hydrochloride, pH 9.0. After 15 min of preincubation it was sprayed with a fivefold-concentrated reaction mixture as used in the OAT assay. After the plate was incubated for a further 30 min at 37 C in the presence of the assay mixture, it was sprayed with 33 mM ortho-aminobenzaldehyde. ACOAT activity was detected by preparing a replica of the wet thin-layer plate on Whatman 3MM paper and cutting the filter paper into 0.5-cm pieces, which were used for determining ACOAT activity in the standard assay (24, 25).

Chemicals. Amino acids were obtained from Fluka (Buchs, Switzerland); bovine serum albumin, urease, Tris (base), and N^2-acetyl-L-ornithine from Sigma; chymotrypsinogen, ovalbumin, aldolase, and cytochrome *c* from Boehringer (Mannheim, Germany); diethylaminoethyl (DEAE)-cellulose (DE-52) from Whatman (Maidstone, England); Diaflo PM-30 ultrafiltration membranes from Amicon (Oosterhout, Holland); and the reagents for polyacrylamide gel electrophoresis from Serva (Heidelberg, Germany). Human hemoglobin A was a gift from M. Landolt. L-Glutamate 5-semialdehyde was derived from N^2-acetyl-L-glutamate 5-semialdehyde, prepared according to a described method (34) by acid hydrolysis in 3.5 M HCl and 100 C for 60 min. 2-Oxo-5-amino-valeric acid was synthesized as described (20). L-[U-^{14}C]ornithine and [1-^{14}C]2-oxoglutarate were received from the Radiochemical Centre, Amersham, England. They were examined for purity and, if necessary, purified by high-voltage paper electrophoresis. The biochemical preparation of N^2-acetyl-L-[U-^{14}C]ornithine will be described in a future communication (R. Voellmy and T. Leisinger, manuscript in preparation).

Enzyme purification. All operations were performed at 4 C. Two 75-ml portions of a 20% (wt/vol) suspension of cells grown in medium P with L-arginine as the only carbon and nitrogen source (large scale cultivation) in buffer A (0.1 M potassium phosphate, pH 7.0, containing 100 μM pyridoxal-5'-phosphate and 1 mM each of 2-oxoglutarate and 2-mercaptoethanol) were subjected to sonic disruption in an MSE 150 W Sonifier by a series of five 1-min exposures. Cellular debris was removed by centrifugation at 20,000 $\times$ *g* for 60 min. The pH of the supernatant solution was adjusted to pH 5.3 by slowly adding 0.1 M acetic acid, and the precipitate was removed by centrifuging at 20,000 $\times$ *g* for 45 min. The pH of the supernatant solution from the initial precipitation was then further lowered to pH 4.6 with 0.1 M acetic acid and the precipitate which was obtained after centrifugation (as above) was suspended in 80 ml of buffer A and dialyzed overnight against 500 volumes of the same buffer. Solid ammonium sulfate (29.5 g/100 ml) was then added to the dialyzed preparation to give 50% saturation, the solution was stirred for an additional 60 min, and the precipitate was removed by centrifugation at 20,000 $\times$ *g* for 45 min. The ammonium sulfate concentration of the supernatant solution was then increased to 80% (23 g/100 ml) and the precipitate obtained by centrifugation (as above) was dissolved in 15 ml of buffer A and dialyzed overnight against 100 volumes of the same buffer. This sample, after addition of 20 mg of blue dextran and sucrose to give a final concentration of 5% (wt/vol), was applied to a column (90 by 5 cm) of Sephadex G-100 equilibrated with buffer A. Elution was carried out with buffer A at a rate of 1 ml per min. Fractions (7 ml) containing high ACOAT activity were pooled, concentrated to 20 ml by ultrafiltration in an Amicon cell with a Diaflo PM 30 membrane, and dialyzed against 100 volumes of 0.1 M potassium phosphate, pH 7, containing 1 mM 2-mercaptoethanol. This preparation was diluted fourfold by the addition of distilled water and was applied to a DEAE-cellulose (DE-52) column (14 by 1.7 cm) equilibrated with 25 mM potassium phosphate, pH 7.0, containing 1 mM 2-mercaptoethanol. Elution was carried out at a flow rate of 1 ml per min with a linear salt gradient from 0.10 to 0.25 M KCl prepared in the buffer described above. Fractions (4.5 ml) were collected in tubes containing 0.5 ml of 1 M potassium phosphate, pH 7.0, 1 mM pyridoxal-5'-phosphate, and 1 mM 2-mercaptoethanol. ACOAT and OAT activity eluted at 0.18 M KCl. The pooled fractions containing enzyme activity were concentrated by ultrafiltration and stored at -20 C without loss of activity over a period of at least 3 months.

Further purification of this preparation was achieved by preparative polyacrylamide gel elcetrophoresis with the buffer system described by Davis (7). Electrophoresis was done with 7.5% acrylamide gels (8 cm in length) in an apparatus from Shandon. After an overnight current flow in gel buffer, samples containing 0.3 to 0.5 mg of protein in 1.0 to 1.5 ml of buffer A with 5% sucrose and 0.05% bromophenol blue as a tracking dye were applied to the gel. Electrophoresis was carried out at a current of 80 mA (at 400 V). The flow rate of the eluting buffer was 0.5 ml per min. Fractions of 1.8 ml were collected in tubes containing 0.2 ml of running buffer with 1 mM pyridoxal-5'-phosphate. The fractions containing enzyme activity were pooled, concentrated by ultrafiltration, and stored at -20 C.

RESULTS

Induction of ACOAT by arginine. Upon addition of L-arginine to a culture of strain PAO 1 growing on medium P with L-glutamate, an increase in the differential rate of ACOAT synthesis was observed (Fig. 1), indicating that L-arginine induced ACOAT. Inclusion of ammonium-sulfate (2 g/l) in the medium had no effect on the induction of ACOAT by L-arginine. As evident from Fig. 1, the rate of enzyme synthesis in a mixture of L-arginine and L-glutamate amounted to about 50% of the rate ob-

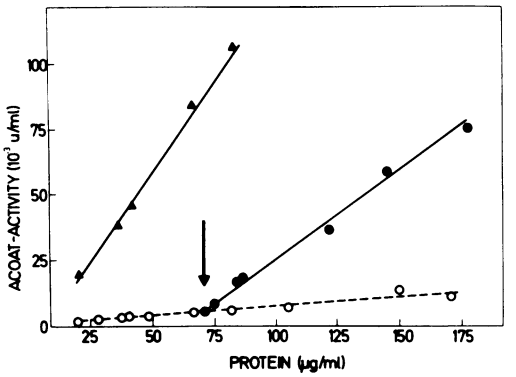

FIG. 1. *Induction of ACOAT by L-arginine. At intervals, 5-ml samples were taken from two Fernbach flasks each containing 1 liter of medium P with L-glutamate, with an exponentially growing culture of strain PAO 1, and immediately subjected to sonication. To determine ACOAT activity in the comparatively dilute cell extracts, the sensitivity of the standard ACOAT assay was increased 10-fold by the use of concentrated reagents. Protein values were obtained by converting the absorbance at 546 nm of the cultures with a calibration curve. After approximately two doublings of cell mass (arrow), 20 ml of 1 M L-arginine-hydrochloride was added to one flask. Symbols: medium P with L-glutamate without L-arginine (○) and with L-arginine (●); control medium P with L-arginine (▲).*

tained on a medium with L-arginine as the sole source of carbon and nitrogen. This observation indicated that the synthesis of ACOAT, in addition to induction by L-arginine, is under control of catabolite repression, thus exhibiting a regulatory pattern typical for a catabolic enzyme. In additional experiments (not shown) catabolite repression of ACOAT was confirmed. Strain PAO 1 was grown on medium P with L-arginine supplemented with either 20 mM L-glutamate or 20 mM citrate or 28 mM glucose (0.5%). Depending on the addition to the L-arginine medium the rate of synthesis of ACOAT decreased by a factor of 2 to 5 from the enzyme level on L-arginine medium without addition.

Purification of ACOAT. In crude extracts of *P. aeruginosa* PAO1, an enzymatic activity catalyzing the transamination of L-ornithine to L-glutamate 5-semialdehyde was present (OAT, EC 2.6.1.13). This OAT activity is induced by arginine and subject to catabolite repression to a similar extent as ACOAT. OAT plays a role in arginine catabolism of many microorganisms (3, 8, 22, 29). The unusual regulatory pattern observed for ACOAT could be explained if the same protein transaminated L-ornithine as well as N^2-acetyl-L-ornithine. We therefore have purified ACOAT from strain PAO 1 assaying for both ACOAT and OAT activity.

In the purification of ACOAT as described above, the following observations were taken into account. For the stability of ACOAT during purification, it was critical to prevent dissociation of pyridoxal-5'-phosphate from the enzyme. In the early purification steps this was achieved by addition of pyridoxal-5'-phosphate to the buffer solutions. During DEAE-cellulose chromatography and preparative gel electrophoresis over 95% of the enzyme activity disappeared due to dissociation of the coenzyme. Almost complete recovery of activity was obtained by incubating the purified preparations in 0.1 mM pyridoxal-5'-phosphate for 10 h at 4 C (at least 40-fold molar excess of pyridoxal-5'-phosphate over enzyme). In crude extracts the ACOAT precipitated upon the addition of acetic acid between pH 5.3 and 4.6. In gel electrofocusing experiments purified ACOAT precipitated as a single band at pH 4.9 to 5.0. Acid precipitation was used in the purification procedure and, although resulting in a lowering of specific activity (Table 1), proved essential for the successful purification of the enzyme.

Table 1 shows that ACOAT and OAT activities are copurified. The enzyme preparation obtained after DEAE-cellulose chromatography was approximately 90% pure as judged by analytical and sodium dodecyl sulfate gel electrophoresis. A portion of this preparation was subjected to further purification by preparative gel electrophoresis. The resulting preparation was homogeneous in analytical (Fig. 2) as well as in sodium dodecyl sulfate gel electrophoresis (Fig. 3) and exhibited both ACOAT and OAT activity in a ratio of 4.2 to 1.0.

During the first three purification steps, the ratio of ACOAT to OAT activity remained constant at a value of approximately 3.2. After gel filtration on Sephadex G-100 this ratio changed to 4.3, the value retained during further purification (Table 1). This shift in the ratio of ACOAT to OAT activity is explained by the elimination of a second enzyme with OAT and ACOAT activity by gel filtration. As shown by the elution profile represented in Fig. 4A, gel filtration leads to the separation of two aminotransferases with different activity ratios. The enzyme eluting as component 2 in Fig. 4A exhibits a ratio of ACOAT to OAT activity of approximately 0.7, whereas the activity ratio of component 1 is approximately 4.3. When crude extracts from noninduced cells grown on medium P with L-glutamate or from induced cells grown with L-arginine were analyzed by gel filtration, similar profiles were obtained (data not shown).

In *Pseudomonas* species a putrescine aminotransferase (EC 2.6.1.29) probably also catalyz-

TABLE 1. *Purification of ACOAT from P. aeruginosa strain PAO 1*

Fraction	Volume (ml)	Total protein (mg)	Sp.act.[a] ACOAT	Sp.act.[a] OAT	Ratio ACOAT/ OAT	ACOAT yield (%)	Purification (-fold)
1. Crude extract	150	1475	1.23	0.36	3.4	100	1
2. Acid fractionation	100	1115	0.89	0.30	3.0	55	0.7
3. Ammoniumsulfate fractionation	15	135	4.83	1.46	3.3	36	4
4. Sephadex G-100[b]	20	20	31.90	7.37	4.3	35	26
5. DEAE-cellulose[b]	18	9	49.10	11.20	4.4	24	40

[a] Expressed as units of enzyme activity per milligram of protein.
[b] Only component 1 of Fig. 4A is used for the results presented.

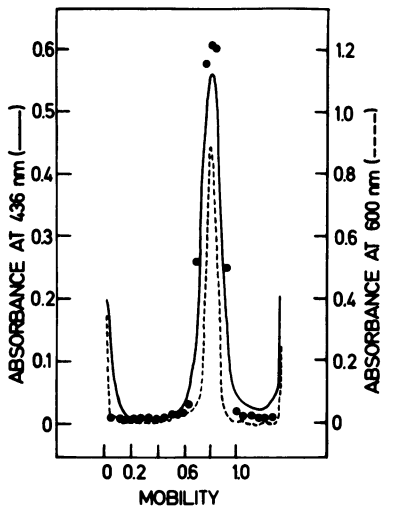

FIG. 2. *Analytical gel electrophoresis of purified ACOAT. Protein scan at 600 nm (-----). scan at 436 nm after OAT activity staining (——), ACOAT activity measured in gel slices (●). Mobility is expressed relative to the front dye bromophenol blue.*

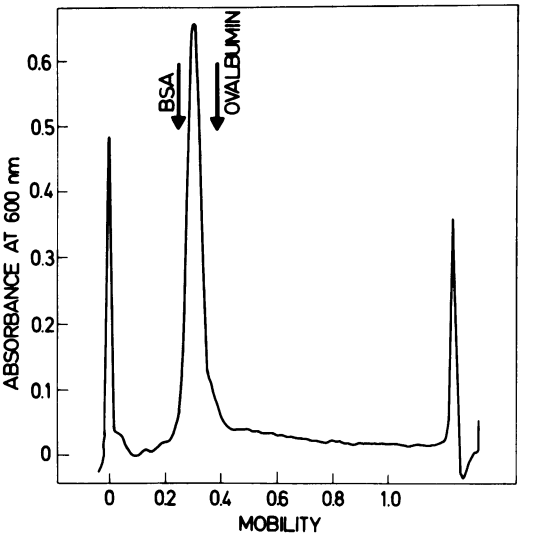

FIG. 3. *Sodium dodecyl sulfate gel electrophoresis of purified ACOAT. Arrows indicate positions of bovine serum albumin and hen ovalbumin in gels subjected to electrophoresis under the same conditions. Mobility is expressed relative to the front dye bromophenol blue.*

ing the transamination of L-ornithine has been described (5). The following lines of evidence indicate that the aminotransferase of component 2 is the corresponding enzyme of *P. aeruginosa* PAO 1. If a crude extract from strain PAO 1 grown on medium P with putrescine was analyzed by gel filtration on Sephadex G-100 (Fig. 4B), one observed a putrescine aminotransferase whose elution volume was identical with the elution volume of component 2 in Fig. 4A. Putrescine aminotransferase as measured in crude extracts was induced about fivefold in cells grown on medium P with putrescine over the activity in cells grown on the same medium with L-arginine. Whereas the ratio of ACOAT to OAT activity in crude extracts of arginine-grown cells (specific activity of ACOAT 1.23 U/mg) was 3.4, this ratio changed to 1.0 in putrescine-grown cells (specific activity of ACOAT 0.3 U/mg). On the basis of these data we have calculated that this change reflects an increase in the level of component 2 enzyme and a corresponding decrease in the level of component 1 enzyme. The ratio of ACOAT to OAT activity of the component 2 enzyme was 0.7 (Fig. 4B), thus corresponding to the activity ratio of component 2 (Fig. 4A). The OAT activities in both component 2 represented in Fig. 4A and component 2 represented in Fig. 4B were inhibited to the same extent by different analogues of amino substrates (Table 2).

Properties of ACOAT. The radioactive product formed from L-[U-^{14}C]ornithine by purified ACOAT was analyzed by high voltage paper electrophoresis. It was identified as L-[U-^{14}C]glutamate 5-semialdehyde. Thus ACOAT catalyzes the ω-transamination of L-ornithine. The pH dependence of purified ACOAT with N^2-acetyl-L-ornithine or L-ornithine as amino donors is represented in Fig. 5. The optima of

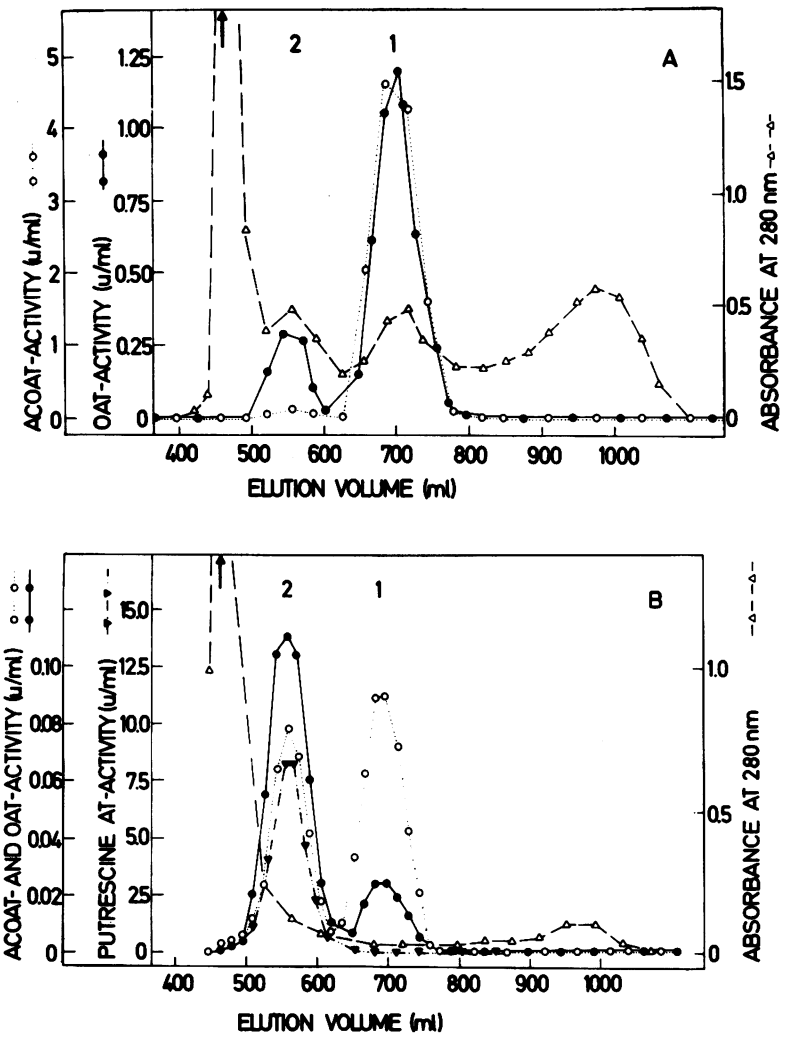

FIG. 4. *Gel filtration of ACOAT on Sephadex G-100. (A) Profile of a partially purified preparation (step 3, Table 1) from cells grown on medium P with* L-*arginine. (B) Profile of a crude extract from cells of a 1-liter culture grown on medium P with putrescine. Absorbance at 280 nm* (Δ), *OAT activity* (●), *ACOAT activity* (○), *putrescine aminotransferase activity* (▼).

the two activities differ by approximately 1 pH unit.

The results of experiments on the kinetics of ACOAT are presented in Fig. 6A as double reciprocal plots of initial velocities versus 2-oxoglutarate concentrations at different concentrations of N^2-acetyl-L-ornithine. K_m values were determined graphically from secondary plots of 1/intercept of primary plot versus 1/s at infinite concentration of the second substrate. A K_m of 1.1 mM for N^2-acetyl-L-ornithine and of 0.7 mM for 2-oxoglutarate was determined. A similar analysis for the OAT reaction was based on the data presented in Fig. 6B. It revealed a K_m of 10.0 mM for L-ornithine and a value of 0.7 mM for 2-oxoglutarate. In the presence of high concentrations of 2-oxoglutarate the OAT reaction was partially inhibited, probably due to the formation of an abortive complex of enzyme with 2-oxoglutarate (29). This inhibition was counteracted competitively by L-ornithine.

Estimations of the molecular weight of ACOAT were done by thin-layer gel filtration with Sephadex G-200 in different buffer systems and with samples from different stages of the purification procedure. In all cases a molecular weight of 110,000 ± 10,000 was obtained (Fig. 7). Estimation of the molecular weight of the

TABLE 2. *Inhibition of OAT activity by amino donor analogues*

Amino donor analogue (25 mM)	Inhibition of OAT activity (%)[a]		
	Pure ACOAT	Amino transferase from component 2 in Fig. 4A	Amino transferase from component 2 in Fig. 4B
	0	0	0
N^2-acetyl-L-ornithine	95	30	30
N^2-acetyl-L-lysine	80		
2,4-Diamino-L-butyrate	50		
Putrescine	45	0	5
5-Aminovaleric acid	40		
L-Arginine	40	0	0
Cadaverine	35		
L-Lysine	15		
4-Aminobutyrate	0	60	55

[a] Assayed under standard conditions using 5 mM L-ornithine.

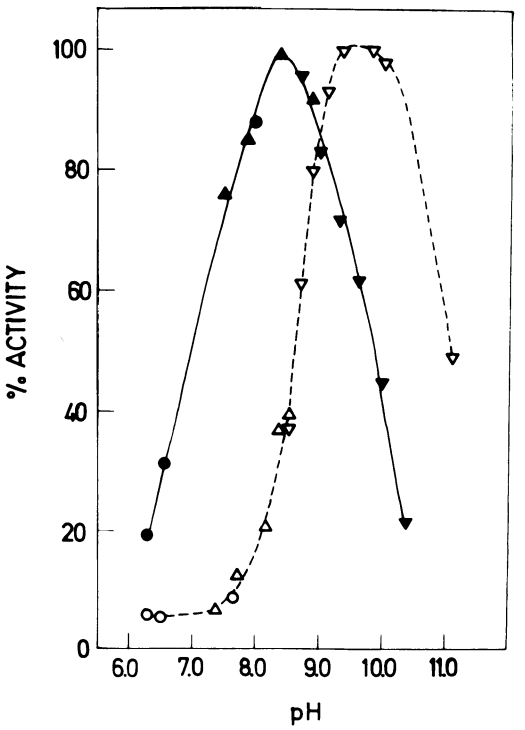

FIG. 5. *pH-dependence of ACOAT activity and OAT activity of pure ACOAT. Assays were performed under standard conditions except for variations of the buffers. Closed symbols represent ACOAT activity, open symbols OAT activity. 0.1 M potassium phosphate (●, ○), 0.1 M Tris-hydrochloride (△, ▲), 0.1 M borate (▼, ▽).*

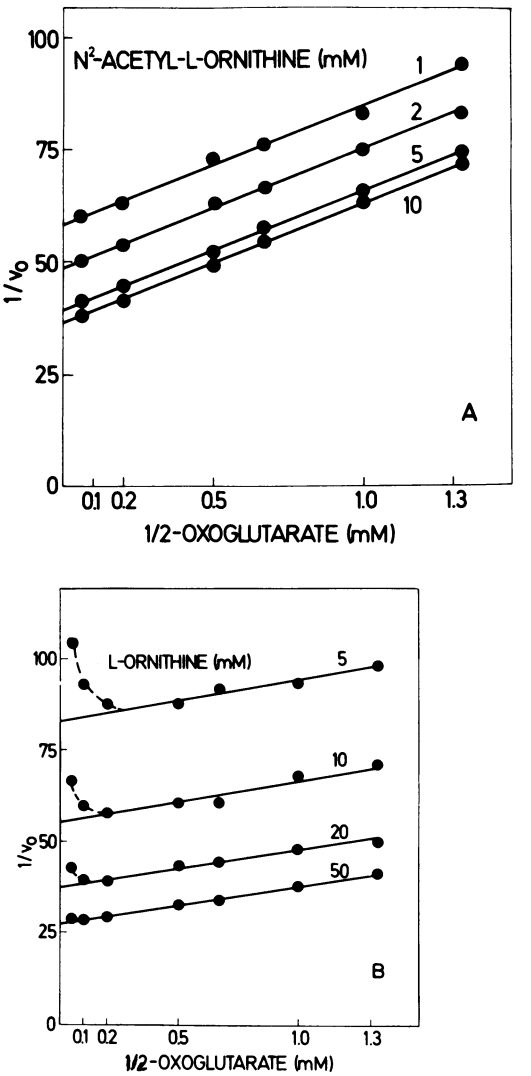

FIG. 6. *Determination of K_m values with pure ACOAT. Assays were performed under standard conditions except for the variations in the substrate concentrations (A) Double reciprocal plot of 1/initial velocity of the ACOAT reaction (relative activities) versus 1/2-oxoglutarate concentrations at different concentrations. (A) Double reciprocal plot of 1/initial velocity of the OAT reaction (relative activities) versus 1/2-oxoglutarate concentrations at different concentrations of L-ornithine.*

enzyme by centrifugation in sucrose gradients. (21) led to a value of approximately 105,000. Electrophoresis of pure enzyme in sodium dodecyl sulfate gels revealed a value of 55,000. This leads to the conclusion that ACOAT consists of at least 2 subunits of equal or similar size.

Substrate specificity. Table 3 shows that, in addition to 2-oxoglutarate, other 2-oxoacids function as amino acceptors in the OAT reaction. Screening for different amino donors was approached by testing different amino donors as inhibitors of the OAT reaction (Table 2). Because of the comparatively high K_m of L-ornithine (10.0 mM), even weak inhibitory effects were expected to be detected in this analysis which was performed at nonsaturating concentrations of L-ornithine. The N^2-acetyl-amino acids, N^2-acetyl-L-ornithine and N^2-acetyl-L-lysine, were strongly inhibitory (Table 2). 2,4-Diamino-L-butyrate, the lower homologue of L-ornithine, gave moderate inhibition whereas L-lysine, the higher homologue, is a weak inhibitor. Putrescine and cadaverine (1,5-diaminopentane), the decarboxylated derivatives of L-ornithine and L-lysine, respectively, were intermediate inhibitors as were 5-amino-valeric acid and L-arginine. Weak inhibition or no inhibition of the OAT reaction (0 to 10% inhibition) was caused by D-arginine, L-citrulline, L-isoleucine L-leucine, L-valine, 4-aminobutyrate, D-ornithine, and L-proline each at 25 mM.

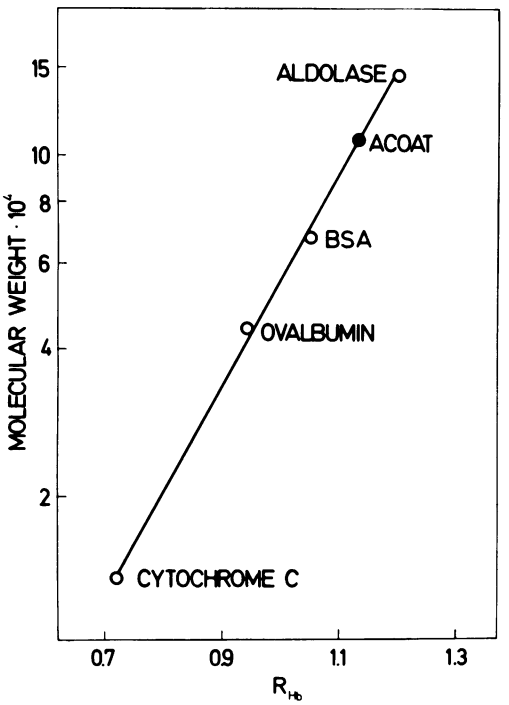

FIG. 7. *Molecular weight estimation by thin-layer gel filtration on Sephadex G-200. The points represent mean values from seven experiments performed with different running buffers (see Materials and Methods). R_{Hb} values indicate migration distances of the different markers relative to human hemoglobin A (64,500 daltons). The markers used were aldolase (149,100 daltons), ovalbumin (43,500 daltons), and cytochrome c (13,400 daltons).*

Some of the amino donor analogues inhibiting the OAT reaction were tested directly as amino donors in the transamination reaction (Table 4). The standard colorimetric assay was used for N^2-acetyl-L-ornithine, L-ornithine, N^2-acetyl-L-lysine, L-lysine, cadaverine, putrescine, and D-ornithine. No detectable activity could be found with the latter four substrates. For the transamination of 2,4-diamino-L-butyric acid and for the calibration of the transamination of N^2-acetyl-L-ornithine, L-ornithine and N^2-acetyl-L-lysine the assay was performed with [1-^{14}C]2-oxoglutarate as the amino acceptor. In preliminary experiments with N^2-acetyl-L-[U-^{14}C]ornithine and [1-^{14}C]2-oxoglutarate as substrates, it was established that the reaction products formed in a 1:1 ratio. Besides N^2-acetyl-L-ornithine and L-ornithine only N^2-acetyl-L-lysine and, to a limited extent, 2,4-diamino-L-butyrate served as substrates of the ACOAT (Table 4).

Catabolic function of ACOAT in vivo. Genetic markers of proline auxotrophy have been mapped in three different loci on the chromosome of *P. aeruginosa* PAO (13). A representative of each group, namely strains PAO 831 (*pro-71*), PAO 853 (*pro-70*), and PAO 879 (*pro-73*), was tested for growth on solid or liquid medium P containing 20 mM L-ornithine and all of the required growth factors at a concentration of 1 mg/ml, excluding proline. Strains PAO 831 and PAO 879 grew on this medium whereas strain PAO 853 was unable to satisfy its proline requirement by L-ornithine. This result is consistent with the observation that strain PAO 853 feeds strains PAO 831 and PAO 879 and reflects an enzymatic lesion of strain PAO 853 between

TABLE 3. *Amino acceptors in the OAT reaction*

Amino acceptor (25 mM)	OAT[a] (% activity)
2-Oxoglutarate	100
Pyruvate	65
2-Oxoadipate	55
2-Oxobutyrate	10
2-Oxovalerate	10
Oxaloacetate	5
2-Oxoisovalerate	5
2-Oxoisocaproate	5
3-Oxoadipate	0

[a] A pure preparation of ACOAT was tested under standard conditions for OAT activity with the different amino acceptors listed.

L-glutamate 5-semialdehyde and L-proline. This is an indication of the in vivo conversion of exogenous L-ornithine to L-glutamate 5-semialdehyde by the OAT reaction.

DISCUSSION

Three separate enzymatic pathways involved in the catabolism of L-arginine have been described in *Pseudomonas*. In one catabolic pathway, arginine is converted to ornithine with citrulline as an intermediate (17, 27); in another, arginine is converted to 4-aminobutyrate via the intermediates of agmatine and putrescine; and in a third pathway to 2-oxo-5-guanidinopentanoic acid which, in turn, leads to 4-guanidinobutyrate (15, 16, 19, 23). The distribution of these pathways in different species of *Pseudomonas* as well as the relative importance of the pathways in vivo is unknown. However it has been shown that *Pseudomonas fluorescens* and *P. aeruginosa* derive energy for the maintenance of motility under anaerobic conditions by degrading arginine to citrulline and ornithine (26). Furthermore the two enzymes specific for the degradation of arginine to ornithine, namely arginine deiminase (L-arginine iminohydrolase, EC 3.5.3.6) and the catabolic ornithine carbamoyltransferase (carbamoylphosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3) are induced by arginine (27).

In this report we present evidence that in *P. aeruginosa* a third step of the latter catabolic sequence is mediated by an arginine-inducible ornithine 5-aminotransferase with a double function in arginine catabolism as well as in arginine biosynthesis.

This enzyme catalyzes the transamination of L-glutamate with N^2-acetyl-L-glutamate 5-semialdehyde when functioning in arginine biosynthesis and probably transaminates L-ornithine with 2-oxoglutarate when participating in the degradation of arginine. Its maximal velocity is higher with N^2-acetyl-L-ornithine and it is therefore named N^2-acetyl-L-ornithine 5-aminotransferase. For the growth of some proline auxotrophic derivatives of *P. aeruginosa* L-ornithine can substitute for L-proline. This demonstrates that L-ornithine is degraded to L-glutamate 5-semialdehyde in vivo. So far we have been unable to select ACOAT-negative mutants of *P. aeruginosa*. The direct conversion of L-ornithine to L-glutamate 5-semialdehyde by ACOAT in vivo is therefore not proven. Also a pathway leading via N^2-acetyl-L-ornithine and N^2-acetyl-L-glutamate 5-semialdehyde to L-glutamate 5-semialdehyde cannot be excluded. It seems, however, very unlikely that N^2-acetylated compounds should function as intermediates in a catabolic pathway. ACOAT from *P. aeruginosa* PAO 1 is induced about 15-fold in cells grown on L-arginine as the sole source of carbon and nitrogen as compared to cells grown on L-glutamate as carbon and nitrogen source. When crude extracts from induced and noninduced cells were analyzed by gel filtration, two peaks with ACOAT as well as OAT activity were detected in both cases. One peak corresponds to putrescine aminotransferase, the other to ACOAT. In both cases more than 95% of the total ACOAT activity was produced by ACOAT.

TABLE 4. *Transamination of different amino substrates by purified ACOAT*

Amino substrate (25 mM)	% Activity at:[a]		Apparent K_m values at pH 9 and 15 mM 2-oxoglutarate (mM)
	pH 8.0	pH 9.0	
N^2-acetyl-L-ornithine	93	100	1.05
L-Ornithine	1	18	10.0
N^2-Acetyl-L-lysine	4	5	2.8
2,4-Diamino-L-butyrate		<1	

[a] The incubation mixture contained 0.1 M Tris-hydrochloride, 25 mM amino substrates, 15 mM 2-oxoglutarate, 0.075 mM pyridoxal-5'-phosphate, and pure ACOAT. Before the addition of enzyme the pH was adjusted to 8.0 or 9.0 at 25 C. For the reaction with the first three substrates the colorimetric assay was used. For calibration of the colorimetric assay and for measurement of the transamination of 2,4-diamino-L-butyrate, an assay with [1-^{14}C]2-oxoglutarate was performed. L-[1-^{14}C]glutamate formed was separated by high voltage paper electrophoresis and assayed for radioactivity in a liquid scintillation counter (see Materials and Methods).

ACOAT from *P. aeruginosa* has been purified to electrophoretic homogeneity. Its molecular weight was estimated as 110,000. The enzyme seems to be composed of two subunits of equal or similar size. With respect to size and subunit composition, the *Pseudomonas* enzyme is similar to the ACOAT from the wild-type strain of *Escherichia coli* W (4), which is repressed by exogenous arginine (1, 4). A secondary mutation in ACOAT-negative mutants (*argD*$^-$) of *E. coli* W leads to the appearance of an ACOAT, specific for N^2-acetyl-L-ornithine and inducible by arginine (4, 32). This mutation occurs at *argM* (2), a locus that is different from *argD*, the structural gene of the wild-type ACOAT. The regulation of ACOAT in an *argD*$^-$*argM*$^-$-strain of *E. coli* W thus is similar to the regulation of ACOAT in *P. aeruginosa*. Whereas the physio-

logical meaning of the induction by arginine is evident for the *Pseudomonas* enzyme, it remains unknown for the ACOAT in *argD*$^-$-*argM*$^-$-strains of *E. coli*. It has been suggested that the inducible ACOAT of these strains might represent an enzyme of a vestigial catabolic pathway (35). Recently OAT activity has been detected in the wild-type strain of *E. coli* W and its identity with ACOAT was demonstrated (J. T. Billheimer and E. E. Jones, personal communication).

The data of a kinetic analysis of purified ACOAT from *P. aeruginosa* PAO 1 are in accordance with a ping pong bi-bi reaction mechanism in Clelands phenomenological classification (6). The K_m observed for N^2-acetyl-L-ornithine was 1.1 mM; for L-ornithine 10.0 mM. ACOATs and OATs from other microorganisms have been studied by different authors (1, 4, 9, 10, 28, 29, 33) and were all shown to be specific for either N^2-acetyl-L-ornithine or L-ornithine. Generally the K_m values of the ACOATS for their amino substrate were lower than the K_m values of the OATs, thus paralleling our observation on the Michaelis constants of the bifunctional ACOAT from *P. aeruginosa*.

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